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VECTOR COMPETENCE OF MOSQUITOES FOR ARBOVIRUSES

Annual Report

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July 30, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7119

University of California
Berkeley, California 94720

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of California; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Berkeley, CA 94720	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-87-C-7119
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102A; PROJECT NO. 3M1 61102BS13; TASK NO. AA; WORK UNIT ACCESSION NO. 054

11. TITLE (Include Security Classification)

VECTOR COMPETENCE OF MOSQUITOES FOR ARBOVIRUSES

12. PERSONAL AUTHOR(S)
Houk, Edward J. and Hardy, James L.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 7/28/87 TO 7/27/88	1988 July 30	47

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	13	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
RA I; Entomology; Vector Competence; Gut Barrier; Genetics; Mosquitoes: Arboviruses; Dengue Fever; Infectious Diseases

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Aedes aegypti mosquitoes are very refractory to peroral infection by dengue 2 (New Guinea C) virus; ID_{50}'s in excess of 7 Log_{10} PFU/ml ingested. Parenteral infection ID_{50}'s are just slightly above 2 Log_{10} PFU/ml.

Binding of western equine encephalomyelitis (WEE) virus to brush border fragments (BBF) from mesenteronal epithelial cells of susceptible (WS) Culex tarsalis was greatly in excess of that to refractory (WR) Cx. tarsalis and Culex pipiens. The binding was specific. There are an estimated 1.7-3.4 x 10^6 binding sites/mesenteronal epithelial cell and the affinity constant is 2.3 x 10^{11} M^{-1}.

Monoclonal antibodies were produced against the BBF of mesenteronal epithelial cells. Three hybridomas (13A5, 13C1 and 20C5) were subcloned and ascites fluid produced. The 13A5 and 20C5 hybridomas are IgG_{2b} subtypes and 13C1 is an IgM.

Further biochemical analysis of BBF proteins revealed that Cx. pipiens closely resembles WR Cx. tarsalis in its 2D electrophoretic pattern. Reversed phase high performance liquid chromatography did not separate the major proteins of interest.

(OVER)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Virginia M. Miller	22b. TELEPHONE (Include Area Code): 301/663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

19. A congenic line of high WEE virus producing (HVP) Cx. tarsalis was derived from low WEE virus producing (LVP) Cx. tarsalis after 13 generations of backcrossing.

I. Summary

Ten geographic strains of the mosquito, *Aedes aegypti* , have been assembled and are currently being maintained in our insectary. Two of these strains (Rockefeller and Jupp) have been compared for their susceptibility to dengue 2 (New Guinea C) virus by the peroral and parenteral has routes. The Rockefeller strain has a peroral ID_{50} of 8.6 $\log_{10}$ PFU/ml and a parenteral ID_{50} of 2.3 $\log_{10}$ PFU/ml. However, we were only able to infect 33% of the Rockefeller strain after feeding on the highest dose of virus available. The Jupp strain has a parenteral ID_{50} of 2.2 $\log_{10}$, virtually identical to that noted for the Rockefeller strain. In terms of peroral susceptibility, the Jupp strain was essentially refractory; 1/27 infected following ingestion of 7.1 $\log_{10}$ PFU/ml. A more efficient means for the peroral infection of *Ae. aegypti* is being sought.

Several methods have been evaluated that will allow us to: 1) perorally infect mosquitoes; 2) assay infected mosquitoes; and 3) determine transmission rates. Thus far, frozen virus stocks from various sources have proven to be unsatisfactory for the infection of *Ae. aegypti* mosquitoes. Plaque assay of triturated mosquitoes in Vero cells by a double overlay method is the best of the plaque assays examined. It would appear that there is essentailly 100% correlation between plaque assay determination of infection and immunofluorescent assay of head squash smears following 10 days of extrinsic incubation at 32°C. Transmission of dengue 2 virus by collecting mosquito saliva in capillary tubes containing borate buffer (pH 9.0), 0.75% bovine albumin and 10% sucrose, which is then frozen at -70°C until plaque assay, has not proven to be very sensitive. However, if the fluid is added directly into tissue culture medium containing BHK-21 cells and amplified for 7 days before plaque assay, the results are much more promising.

The binding of western equine encephalomyelitis virus to brush border fragments isolated from the mesenteronal epithelial cells of the WS strain of the mosquito, *Culex tarsalis*, was found to be specific. The binding could be saturated and bound radiolabelled was competitively displaced by unlabelled virus; two basic criteria for specific binding. Scatchard analysis of the binding data resulted in an estimate of the number of binding sites/mesenteronal epithelial cell at 1.7-3.4 x 10^6. The affinity constant was determined to be 2.3 x 10^{11} M^{-1}.

Three hybridomas (13A5, 13C1 and 20C5) derived from mice immunized with Triton X-100® solubilized brush border fragments from the mesenteronal epithelial cells of *Cx. tarsalis* were subcloned twice to insure their monoclonality. Ascites fluid was produced and collected. Two of these proved to be IgG_{2b} isotypes (13A5 and 20C5). The third (13C1) was an IgM isotype.

The production of ascites fluid had very mixed results. In excess of 200 ml of fluid was obtained from 24 mice inoculated with 20C5 cells. In addition, a test of the effects of hydrocortisone on enhancing ascites fluid production was encouraging. One mouse treated with hydrocortisone yielded approximately 50 ml of ascites fluid. Approximately 30 ml of ascites fluid was collected from mice inoculated with 13C1 cells. This fluid will be used without further treatment since the antibody isotype is IgM. The 13A5 cells yielded a rather disappointing 35-40 mg of monoclonal antibody protein following ammonium sulfate fractionation and protein A column chromatography. This amount of antibody is not sufficient for production of a column for antigen isolation. However, these cells are currently growing in a bioreactor, as part of a test for the campus Hybridoma Laboratory, and initial results are very encouraging.

Two dimensional electrophoretic analysis of the brush border fragment protein pattern from the mesenteronal epithelial cells of *Culex pipiens* demonstrated a marked similarity to that observed for WR and LVP *Culex tarsalis* . Two high molecular weight proteins predominate the pattern: one of approximately 150 kDal and another of approximately 100 kDal.

Reversed phase high performance liquid chromatography was examined as a means of separating brush border proteins. The most hydrophobic (i.e., membrane) proteins are eluted as a group consisting of 3 or sometimes 4 distinct peaks. The nonionic detergents, n-octyl glucoside and n-octyl thioglucoside, are much better solubilizing agents, when compared to Triton X-100, for use in HPLC since they are not inherently absorbant in the ultraviolet range used for protein detection.

Thirteen generations of backcrossing experiments have yielded a line of *Culex tarsalis* that is homozygous for the trait of high WEE virus production (HVP). This line was derived exclusively from low WEE virus producing (LVP) parents. The HVP and LVP lines are expected to differ only in their ability to modulate WEE virus replication. Thus, these 2 lines are termed congenic.

Foreword

In conducting research using animals, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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II. Introduction

If a putative mosquito vector species feeds on an arbovirus infected vertebrate host, the potential exists for the vector to become infected with and to subsequently transmit the virus to another susceptible vertebrate host. The probability of vector infection depends on a number of intrinsic factors which our research program has been able to delineate through many years of studying the mosquito, *Culex tarsalis* , and the alphavirus, western equine encephalomyelitis (WEE) virus (Hardy et al., 1983). The measure of relative susceptibility of a vector population has been referred to as vector competence.

The mechanisms governing the vector competence of mosquitoes for WEE virus have been elucidated using two different mosquito models. An interspecific model, *Cx. tarsalis* and *Culex pipiens* , has allowed us to examine the mesenteronal infection barrier (MIB) and to establish that, in this system, the barrier is governed by receptor sites for the attachment of WEE virions to microvillar membranes (Houk et al., 1986; Houk et al. In manuscript). An intraspecific model system, WEE virus susceptible (WS) and WEE virus refractory (WR) genetic strains of *Cx. tarsalis*, expresses a MIB component and a second viral regulatory mechanism often referred to as modulation (Murphy 1975; Murphy et al. 1975; Hardy et al. 1983; Kramer et al., In manuscript). It would appear that expression of the MIB in WR *Cx. tarsalis* is a consequence of the absence of receptor sites on the microvillar membranes (Houk et al., In manuscript), analogous to the interspecific model. The production of monoclonal antibodies to mivrovillar proteins and further analysis of the binding of radiolabelled WEE virions to microvillar membranes are examined in this progress report.

The modulation of alphaviruses by WR *Cx. tarsalis* has been exploited through the genetic selection of a low WEE virus producing (LVP) strain of *Cx. tarsalis* . This strain was obtained by selecting those individual mosquitoes that produced less than 4.0 $\log_{10}$ PFU of WEE virus following parenteral infection. It has been established through the use of DNA dependent RNA transcription inhibitors (i.e., actinomycin D and α-amanitin) that modulation is a property of the infected mosquito and not related to infecting virus (Houk et al., In manuscript). In preparation for molecular studies, we have produced a strain of high WEE virus producing (HVP) *Cx. tarsalis* that is congenic to the original LVP strain. The congenic selection scheme and its results are reported herein.

Currently, our focus of attention is on the mosquito, *Aedes aegypti* , and the flavivirus, dengue. The literature (Gubler et al., 1982) suggested that this particular system would

perhaps be mosquitoes. We have begun the screening of a number of geographic strains of *Ae. aegypti* in order to determine their relative infection rates following peroral and parenteral infection. We are examining the sensitivity of various methods to determine if a mosquito is infected and if that mosquito can transmit the virus. These experiments are reported in detail within this report.

III. Mosquito Colonies Maintained for Vector Competence Studies

A number of mosquito species and strains that are relevant to our studies on the vector competence of mosquitoes for both alpha- and flaviviruses are maintained in our insectary. In addition, a number of these strains represent unique genetic strains of *Cx. tarsalis* that are exemplary of barriers associated with the inability to become infected with and the subsequent failure to transmit WEE virus.

An excessive number of geographic strains of *Ae. aegypti* are being maintained at present until their susceptibility to peroral and parenteral infection with dengue viruses has been determined. The number of geographic strains maintained in our insectary will be reduced when a strain(s) is identified that is a likely candidate for selection of refractory and susceptible genetic lines.The goal of these studies is the production of genetic strains of *Ae. aegypti* that exhibit specific barriers to the infection and transmission of flaviviruses, analogous to our *Cx. tarsalis* models for alphaviruses .

TABLE 1. Mosquito colonies maintained for vector competence studies.

SPECIES	COLONY DESIGNATION
Aedes aegypti	Jupp, Jakarta, Puerto Rico, Rexville, Rockefeller, Ogbomosho, Waco, Bangkok, Vero Beach, Miami
Aedes dorsalis	Ft. Baker
Culex peus	Grasshopper Slough
Culex pipiens	Poldervaart
Culex quinquefasciatus	BFS
Culex tarsalis	Chico; Ft. Collins; Knights Landing; Poso Creek; KL-HVP; Manitoba; PC-HVP; WR-1 (FC-KL); WR-1 LVP; WR-2 (FC-KL-C); WS-2 (KL); WS-3; Yuma; Y-HVP

IV. Studies on the Vector Competence of *Aedes aegypti* for dengue viruses

A. Developmental studies

The New Guinea C strain of dengue 2 (Den2) virus (kindly provided by Dr. S. Kliks, University of California, Berkeley CA) was selected for initial studies of peroral and parenteral infection of geographic strains of *Ae. aegypti* . In these studies, various methods have been evaluated to determine the best methods for infecting mosquitoes and measuring infection and transmission rates. Further, we have been able to obtain anti-dengue virus immune mouse ascitic fluid (Dr. E. Henchal, WRAIR, Washington DC) to allow detection of potential dengue viral infections by immunofluorescence assay (IFA) in mosquitoes and by *in situ* enzyme immunoassay (EIA) of infected cell cultures.

1. *Evaluation of methods to assay mosquitoes for dengue viral infection*

Two different methods, plaque assay and immunofluorescent assay (IFA), have been evaluated for detection of viral infections in *Ae. aegypti* . Plaque assays were done on homogenates of individual females in two different vertebrate cell lines: BHK-21 and Vero cells and IFA was done on head squash smears and dissected salivary glands.

Plaque assay for Den2 virus is not very has not been particularly sensitive. Several modifications of the basic plaque assay technique were examined. These involved evaluation of plaquing efficiency in both Vero or BHK-21 cells, the effects of overlay medium (i.e., methyl cellulose or agar), a double overlay modification. In addition, the technique of staining plaques, with either neutral red or naphthol blue black, for plaque differentiation was evaluated.

The titer of Den2 virus in those cells incubated for 7 days and stained with naphthol blue black was higher in BHK-21 cells ($10^{5.7}$/ml) than in Vero cells ($<10^{5.0}$/ml). Following a double overlay protocol in Vero cells, measured virus titers were slightly increased. Den 2 titers were not effected by whether the first overlay methyl cellulose ($10^{6.5}$ PFU/ml) or agar ($10^{6.4}$ PFU/ml). However, following an agar first overlay, the plaque morphology appeared to be more distinct than the other methods. An additional measure of safety is found with the two overlay method, in that handling of media containing high titers of virus is eliminated.

2. *Evaluation of methods to infect mosquitoes perorally with dengue viruses*

The feeding suspension for infecting mosquitoes with dengue viruses was that of Gubler and Rosen (1976). Supernatant derived from Den2 infected *Ae. albopictus* (C6/36) cells is mixed with equal volumes of washed human erythrocytes and 10% sucrose. Gubler and Rosen (1976) allowed adult females to feed on small droplets of infectious blood placed directly on the mesh covering of the holding containers. As a comparison, gauze pledgets were soaked with this mixture and placed on the mesh covered tops of pint ice cream cartons containing mosquitoes to be infected. Mosquitoes to be fed on this infectious bloodmeal were deprived of a sucrose source for 24-48 hrs and water for 24 hr.

The droplet method allows a greater surface area for feeding, using a smaller quantity of infectious blood, and thus, reduces the competition for feeding sites between the 50-70 mosquitoes confined within the pint carton cage. A disadvantage to the droplet method is a more rapid dehydration of the infectious bloodmeal compared to the gauze pledget. However, properly starved mosquitoes are most often finished feeding within 30 min, negating the effects of dehydration on the infectious bloodmeal.

3. *Evaluation of in vitro transmission systems for measuring transmission rates*

Transmission rates are determined by removing the legs and wings from the potentially infected female mosquitoes and inserting their proboscis into a capillary tube containing 10% sucrose in BA/BSB (0.75% bovine albumin (w/v); 0.05 M boric acid, 0.12 M NaCl; pH 9.0). A period of time was allowed for salivation into, and ingestion of, the fluid. The original level of liquid in the capillary tube is marked and after a measurable quantity of fluid ha· been imbibed (e.g., at least 3 mm), the fluid is expelled into MEM tissue culture medium and rozen at -70°C. After it has been determined which mosquitoes became infected, the complementary salivary secretion samples are then assayed to establish the transmission rate.

The transmission rate was essentailly zero when using the above protocol. As an alternative, freshly collected salivary secretions in BA/BSB were inoculated directly into MEM in a 96-well plate and BHK-21 cells added. The cells were allowed to replicate any dengue virus present in the original inoculum for 7 days. The supernatants from these cells were then plaque assayed to determine transmission rates. The transmission rates were still extremely low but this method yielded a measurable improvement compared to the method of direct plaquing.

It would appear that transmission rates might be determined with higher sensitivity by the intrathoracic inoculation of mosquitoes. This method involves intrathoracic inoculation of 5 mosquitoes with the potentially infectious fluid containing the mosquito salivary secretions, collected by the capillary tube method. After an incubation period of 10 days at 27°C, plaque assays or head squash smears will be evaluated to determine the percent transmission. This method will be evaluated when peroral infection rates have been maximized.

B. Characterization of geographic strains of Aedes aegypti for their vector competence with dengue viruses - Variations in peroral and parenteral susceptibility

The peroral and parenteral infection rates for different geographic strains of *Ae. aegypti* have been, and will continue to be, measured. A population of mosquitoes that is 100% infected by the peroral route after feeding on a "moderate" dose of Den2 virus would be ideal for the initiation of genetic selection of refractory and susceptible strains of *Ae. aegypti* . Parenteral infection allows us to measure two parameters: comparative infection rates by this route and transmission rates for infected mosquitoes. If peroral infection rates are very low, parenteral infection would be the only feasible means of determining threshold concentrations for transmission.

Gubler et al. (1979) reported that geographic strains of *Ae. aegypti* varied significantly in their ability to become infected with dengue viruses. Other studies have included geographic strains of *Ae. aegypti* in their attempts to document variations in the vector competence of mosquito strains and species with various strains and serotypes of dengue viruses (Gubler et al., 1982; Rosen et al. 1985). We are attempting to establish genetic strains of *Ae. aegypti* that are either highly susceptible or highly refractory to peroral infection with Den2 virus. To accomplish this task, we have attempted to establish susceptibility profiles for the various geographic strains of *Ae. aegypti* listed in Table 1.

Our initial experiment was to determine an ID_{50} for the Rockefeller strain of *Ae. aegypti* following parenteral infection (Table 2). The rationale for the choice of this particular strain was that it had been reported to be one of the most susceptible mosquito strains to peroral Den2 infection (Rosen et al., 1985), and thus, might provide a measure of the efficiency of infection to be expected. This strain was 100% infected following parenteral inoculation with 3.9 $\log_{10}$ PFU/ml Den 2 virus and 7 days of extrinsic incubation (EI) at 32°C. One observes a slight increase in the percent infected at 1.9 $\log_{10}$ PFU/ml if the period of EI is increased to 10

days. A graphic solution of the data from Table 2 yields an ID_{50} of 2.5 $\log_{10}$ after 7 days EI and 2.3 $\log_{10}$ after 10 days EI (Fig. 1). This decrease in ID_{50} with time is simply a reflection of the increased probability of detecting an infected mosquito as the EI period is increased (Mitchell et al., 1987).

TABLE 2. Susceptibility of the Rockefeller strain of *Aedes aegypti* to parenteral infection with dengue 2 (New Guinea C) virus.

	Days extrinsic incubation*			
	7		10	
Virus titer** ($\log_{10}$ PFU/ml)	Percent infected (No. positive/tested)	Virus titer§ Mean(Range)	Percent infected (No. positive/tested)	Virus titer Mean(Range)
3.9	100(20/20)	5.3(4.1-6.0)	100(15/15)	5.5(4.2-6.1)
1.9	30(6/20)	5.3(4.4-5.8)	38(8/21)	5.3(4.1-6.5)
-0.9	0(0/20)		0(0/14)	

*Mosquitoes were incubated at 32°C following parenteral infection.
**Stock virus prepared in C6/36 *Aedes albopictus* cells was diluted in PBS and 0.17 µl inoculated intrathoracically into individual female mosquitoes. The titer of the inoculum at each dilution was determined by plaque assay in Vero cells.
§Virus titers for infected mosquitoes were determined by plaque assay in Vero cells.

TABLE 3. Susceptibility of the Rockefeller strain of *Aedes aegypti* to peroral infection with dengue 2 (New Guinea C) virus.

	Days extrinsic incubation*			
	10		14	
Virus Titer** ($\log_{10}$ PFU/ml)	Percent infected (No. positive/tested)	Virus titer§ Mean(Range)	Percent infected (No. positive/tested)	Virus titer Mean(Range)
7.1	25(4/20)	5.4(4.7-5.8)	33(2/7)§§	5.4(4.8-6.0)
5.3	0(0/20)			
4.4	0(0/20)			

*Mosquitoes were incubated at 32°C following peroral infection.
**Stock virus prepared in C6/36 *Aedes albopictus* cells was diluted with equal volumes of outdated human blood collected for transfusion and 10% sucrose. Mosquitoes were exposed to gauze pledgets soaked with this mixture for approximately 1 hr. The titer of the inoculum at each dilution was determined by plaque assay in Vero cells.
§Virus titers for infected mosquitoes were determined by plaque assay in Vero cells.
§§One additional mosquito tested positive by IFA on head squash smear, salivary glands and mesenteron.

Peroral infection proved to be more difficult than anticipated with the Rockefeller strain of *Ae. aegypti* (Table 3). At the highest concentration we were able to feed, 7.1 $\log_{10}$ PFU/ml, only 25% of the mosquitoes were positive after 11 days of EI at 32°C. The infection

rate increased to 33% when the EI period was increased to 14 da. A similar response has been reported by Gubler and Rosen (1976). In their study, the percent infected *Ae. albopictus* was assayed through IFA of salivary glands. Peroral infection of female mosquitoes was by feeding on droplets of blood composed of equal parts of viral suspension, washed human erythrocytes and 10% sucrose. The exact titer of the infectious bloodmeal was not reported. However, it was found that at 7 days no infected salivary glands could be detected, 33% were infected at 10 days, 71% at 14 days and 100% at 28 days EI at 32°C.

The oral ID_{50} for the Rockefeller strain was graphically determined to be 9.8 $\log_{10}$ after 11 days of EI and 8.6 $\log_{10}$ after 14 days of EI (Fig. 2). Again the apparent decrease in ID_{50} following increased EI reflects an increased probability of detecting a positive mosquito by extending the EI period. The ID_{50} for Den2 might be determined by the means in which the virus is presented to the mosquito vector. It has recently been demonstrated that feeding suspensions for several viruses are more infectious if the feeding suspension is prepared on the day of feeding with virus obtained on the same day from infected cell cultures (Miller and Ballinger, 1988).

The Jupp strain of *Ae. aegypti* was also examined for its susceptibility to parenteral (Table 4) and peroral susceptibility (Table 5) to Den2 viral infection. Based on the results with the Rockefeller strain (Table 2), the Jupp strain was inoculated with a narrower range of Den2 viral titers (i.e., 2.9, 1.9 and 0.9 $\log_{10}$ PFU/ml). Of the Jupp females parenterally inoculated with a dose of 2.9 $\log_{10}$ PFU/ml, 95% were infected after 7 da EI and 85% after 10 da EI. Thirty five percent of the inoculated females became infected when inoculated with 1.9 $\log_{10}$ PFU/ml and approximately 10% when inoculated with 0.9 $\log_{10}$ PFU/ml. Graphic determination of the parenteral ID_{50} for the Jupp strain of *Ae. aegypti* yields 2.2 $\log_{10}$ PFU/ml (Fig. 3).

The Jupp strain was almost totally refractory at the maximum dose of Den2 virus fed (Table 5). A single mosquito was found to be infected at 10 days EI and none positive following 14 days EI after feeding on 7.1 $\log_{10}$ PFU/ml. Refractoriness is the usual case and not the exception when dealing with *Ae. aegypti* and dengue viruses. Gubler et al. (1982) reported a maximum infection of 60% for a Miami strain but only 5.6% of a Gambian strain of *Ae. aegypti* following ingestion of 7.6 $\log_{10}$ MID_{50} (the titer required to infect 50% of inoculated mosquitoes) of Den2 virus.

TABLE 4. Susceptibility of the Jupp strain of *Aedes aegypti* to parenteral infection with dengue 2 (New Guinea C) virus.

	Days extrinsic incubation*			
	7		10	
Virus titer** (log_{10} PFU/ml)	Percent infected (No. positive/tested)	Virus titer§ Mean(Range)	Percent infected (No. positive/tested)	Virus titer Mean(Range)
2.9	95(19/20)	5.4(3.3-6.2)	85(17/20)	5.1(4.2-6.1)
1.9	35(7/20)	≥4.7§§	35(7/21)	5.3(4.1-6.5)
0.9	10(2/20)	4.8(4.4-5.3)	8(1/12)	4.4

*Mosquitoes were incubated at 32°C following parenteral infection.
**Stock virus prepared in C6/36 *Aedes albopictus* cells was diluted in PBS and 0.17 µl inoculated intrathoracically into individual female mosquitoes. The titer of the inoculum at each dilution was not determined by plaque assay but estimated to be roughly equivalent to that of the Rockefeller strain experiment.
§Virus titers for infected mosquitoes were determined by plaque assay in Vero cells.
§§Exact titer was not determined.

TABLE 5. Susceptibility of the Jupp strain of *Aedes aegypti* to peroral infection with dengue 2 (New Guinea C) virus.

	Days extrinsic incubation*			
	10		14	
Virus titer** (log_{10} PFU/ml)	Percent infected (No. positive/tested)	Virus titer§ Mean(Range)	Percent infected (No. positive/tested)	Virus titer Mean(Range)
7.1	5(1/20)§§	5.0	0(0/7)	
5.3	0(0/20)			
4.4	0(0/20)			

*Mosquitoes were incubated at 32°C following peroral infection.
**Stock virus prepared in C6/36 *Aedes albopictus* cells was diluted with equal volumes of outdated human blood collected for transfusion and 10% sucrose. Mosquitoes were exposed to gauze pledgets soaked with this mixture for approximately 1 hr. The titer of the inoculum at each dilution was determined by plaque assay in Vero cells.
§Virus titers for infected mosquitoes were determined by plaque assay in Vero cells.
§§One additional mosquito tested positive by plaque assay with a titer of 2.3 log_{10}, but was IFA negative for both head squash smear and salivary glands.

It seems quite apparent that in order to be able to embark on a course to select both susceptible and refractory strains of *Ae. aegypti* to dengue viruses one must be able to infect a sufficient percentage of the mosquitoes ingesting virus. The experiments reported herein indicate only 33% of the Rockefeller strain infected following ingestion of the highest titer of DEN2 virus available to us at this time. Attempts will be made to increase the titer fed upon by using the modifications of Miller and Ballinger (1988). These investigators used the entire

population of C6/36 *Ae. albopictus* cells concentrated into 1 ml of the cell supernatant. The cell suspension (1.0 ml) was diluted into a feeding suspension of washed human red blood cells(4.0ml), fetal bovine serum (4.5 ml) and sucrose (0.5 ml of a 50% solution). The titer of this feeding solution was found to be in excess of 8.0 $\log_{10}$ MID_{50} per ml. The Amphur strain of *Ae. aegypti* was found to be approximately 100% infected following ingestion of maximal titers of either the parental (PR-159) or vaccine (S-1) strains of DEN2 virus. Results such as these are most encouraging and would indicate that finding ways to increase the titer of dengue viruses in the artificial bloodmeals is a prerequisite for starting genetic selections for susceptible and refractory strains of *Ae. aegypti* .

V. Vector Competence Studies on *Culex tarsalis* and Western Equine Encephalomyelitis Virus

A. Mesenteronal infection barrier

The mesenteronal infection barrier (MIB) is the first in a series of membrane associated barriers with which an infecting virus must contend before successful infection of a mosquito vector can occur (Hardy et al., 1983). Recent studies (Hardy et al., 1983; Houk et al., 1986) have suggested that the molecular basis of the MIB resides with an absence or a modification of viral receptor sites along the microvillar surface of mesenteronal epithelial cells. We have attempted to establish the involvement of receptor sites in the infection of mesenteronal epithelial cells by examining the binding of radiolabelled virus to isolated brush border fragments (BBF) from both susceptible and refractory mesenteronal epithelial cells (Houk et al., 1986). In addition, we have initiated studies to produce monoclonal antibodies to BBF from both susceptible and refractory *Cx. tarsalis* with hopes of discovering an antibody specific for the viral receptor.

1. *Binding assays*

We had completed a number of studies of the binding between radiolabeled WEE virus and BBF isolated from susceptible (WS or HP) or refractory (WR, LP or *Cx. pipiens*) strains or species of *Culex* mosquitoes at the beginning of this contract year. We suggested two criteria needed to be satisfied to prove the specificity of the interaction between WEE virus and BBF from susceptible mosquitoes: saturation of binding and competitive displacement. It was proposed that these two experiments would be completed during this contract year and such has been the case.

BBF were isolated according to the methods described by Houk et al. (1986). Vero cells were grown in a 500 ml microcarrier spinner culture until the beads were confluent. The culture was washed 3X in PO_4-free MEM and the cells allowed to incubate for 3 hr in order to deplete intracellular PO_4 pools. Actinomycin D (1μg/ml) and WEE virus (1 MOI) were added to the cultures. Following a 1 hr adsorption period, ^{32}P-orthophosphate (10 mCi) was added to the culture vessel and labeling of virus was carried out overnight. The microcarrier beads were pelleted by centrifugation at 1000xg - 10 min, rinsed in a small volume of PO_4-free medium, centrifuged again and the supernatants combined. The supernatant was further clarified by centrifugation at 12,000xg - 30 min. The supernatant was made 8% in polyethylene glycol 6000 (PEG) and 2.3% NaCl and the virus allowed to precipitate at 5°C overnight with continuous mixing. The PEG precipitated virus was collected by centrifugation at 27,000xg - 45 min, resuspended in approximately 6 ml of TNE and layered directly onto 4 Nycodenz® step gradients (1.3 ml each of 30, 25, 20% Nycodenz® (w/v) in binding buffer[0.15 M NaCl; 0.01M phosphate buffer, pH 7.2] in SW50.1 tubes). The gradient becomes linear during the 18 hrs of centrifugation at 45,000 RPM. The virus band is found approximately 75-85% of the distance through the gradient. The virus band is removed by drop- wise collection through side puncture of the centrifuge tube. The ^{32}P-virus in Nycodenz®/binding buffer was used directly in binding assays without any indication of interference by the Nycodenz®.

The quantities of virus and BBF were estimated by protein determination. The appropriate quantites of each were mixed in a microfuge tube and the volume was brought to 1 ml with 0.75% BSA in binding buffer. Binding was allowed to occur during a 1 hr incubation at the appropriate temperature, generally 20-22°C (i.e., room temperature). Virus bound to BBF was pelleted at 27,000xg - 30 min the supernatant was decanted, the inside of the microfuge tube and the surface of the pellet were gently rinsed and the virus/BBF pellet re-pelleted at 27,000xg - 30 min. The supernatant was decanted and the pellet solubilized in 100 μl of 1% sodium dodecylsulphate by Cerenkov counting.

The binding sites on BBF from WS *Cx. tarsalis* can be saturated. The amount of WEE virus bound at saturation is approximately 4.0 μg/μg BBF from the curvilinear line of best fit (r=0.96; 3rd degree polynomial) for the combined data from 5 different saturation experiments (Fig. 4). Scatchard analysis of the data from these same 5 experiments (r=0.76; $t_{0.01}$=2.90<<4.8) revealed that saturation of binding sites (i.e., the x-axis intercept) occurred at 5.9 μg WEE virus/μg BBF (Fig. 5a). If one assumes that the estimated molecular weight for WEE virus is approximately that reported for Semliki Forest virus [65 x 10^6 daltons (Fries and Helenius, 1979)], this translates into 5.5 x 10^{10} binding sites/μg BBF. The affinity

constant (K_a) as determined from the slope of the Scatchard plot was calculated to be 3.4 x 10^{-2} μg^{-1} (2.3 x 10^{11} M^{-1}).

To determine the number of binding sites per mesenteronal epithelial cell, it was estimated that the lumenal surface area of a midgut is approximately 3 mm^2 and that each mesenteronal epithelial cell is 10 μm in diameter. This results in the calculation of the number of cells per mesenteron as approximately 10,000. Further, we obtain an average of 0.3-0.6 μg BBF protein/mesenteron. Thus, the calculated number of binding sites/μg BBF is 5.5 x 10^{10} and the approximate number of WEE viral binding sites per cell is 1.7-3.4 x 10^6.

Scatchard analysis of the negative control groups was somewhat scattered (Figs. 5b,c). *Culex pipiens* BBF revealed some specific affinity toward WEE virus as exemplified by the intersection of the x-axis by the line of best fit for all three experiments (Fig. 5b). The data from the three experiments was so divergent that common analysis was impossible. WR *Cx. tarsalis* revealed little, if any, affinity for WEE virus (Fig. 5c). The lines of best fit for the two experiments were essentially parallel to the x-axis indicating a constant ratio of virus bound/free virus; a totally nonspecific interaction.

Competitive binding studies revealed a significant competition between labeled and unlabeled WEE virions for attachment sites on BBF (Fig. 6). In the first analysis, we compared the competition of labeled and unlabeled virus for binding sites on the BBF of both WS *Cx. tarsalis* and *Cx. pipiens* (Fig. 6a). Since net radiolabelled virus bound to WS *Cx. tarslais* BBF is less than that bound to *Cx. pipiens* BBF, one might conclude that more specific binding sites are involved in binding competition in the former. As above, if one uses *Cx. pipiens* as a measure of nonspecific binding, then the difference between WS *Cx. tarsalis* and *Cx. pipiens* should represent competition for specific binding sites. The lines of best fit (r=0.90; 0.99; and 1.00) for the three different experiments reveal specific competitive displacement of labelled virus by unlabelled virus (Fig. 6b).

The specific binding of an arbovirus to BBF from mesenteronal epithelial cells had not been previously investigated. Investigators have previously concentrated on the interaction of virions with vertebrate cells, invertebrate cells or liposomes. Mooney et al. (1975) found that Sindbis virus would bind to liposomes containing only phospholipids and cholesterol. This observation prompted the suggestion that direct interaction of virions with the lipid components of the host plasma membrane might best explain the reception of arboviruses by a very wide host range, that includes both vertebrates and invertebrates. Helenius et al. (1978)

suggested that, in human and murine cell lines, histocompatibility antigens were the specific receptors for Semliki forest (SF) virus; HLA-A and HLA-B for human and H-2K and H-2D for murine cells. It was further suggested that since histocompatibility antigens are found on essentially all mammalian cells that these antigens were also prime candidates for the universal receptor for arboviruses (Helenius et al., 1978). This was subsequently refuted when studies with human cell lines that had modified, or lacked, histocompatibility antigens in their plasma membranes were shown to be quite susceptible to SF virus infection (Oldstone et al., 1980). Further, Maassen and Terhorst (1981) revealed through cross-linking studies that Sindbis virus was bound to a 90,000 molecular weight cell-surface protein in two lymphoblastoic cell lines. This protein was distinct from both HLA-A and HLA-B in immunological and cross-linking studies and was suggested to be at, or near, the binding site for Sindbis virus on the cell surface.

Physicochemical studies of arbovirus binding to vertebrate and invertebrate cells have been reported. Fries and Helenius (1979) examined the binding of SF virus to 5 different vertebrate cell lines. They found that the number of receptor sites for SF virions was in the range of 2-5 x 10^4/cell. They were not able to achieve saturation of binding with virions. However, with purified 29S octamer complexes of virus surface glycoproteins, saturation of binding sites on HeLa cells was attained. Under these conditions, the number of receptor sites was found to be 1.8 x 10^6/cell. The affinity constant (K_a) was in the range of 3-5 x 10^{10} M^{-1}. Smith and Tignor (1980) examined the binding of neurovirulent and nonvirulent strains of Sindbis virus to several vertebrate and two insect cell lines. Saturation of binding to all cell lines was obtained. It was demonstrated that cultured neuronal cells had many more receptors for the neurovirulent strain (1.3 x 10^6) than for the nonvirulent strain (5 x 10^4). The average number of receptors for the neurovalent strain on susceptible cell lines was 1.5 x 10^6/cell, while a totally refractory lepidopteran cell line had 1 x 10^5/cell.

Arcus et al. (1983) reported initial studies that indicated that a differential existed in the ability of WEE virus to bind to BBF from susceptible WS *Cx. tarsalis* as compared to refractory WR *Cx. tarsalis* and refractory *Cx. pipiens*. The parameters for binding in these initial studies were basically those of Smith and Tignor (1980), with no attempt at optimization. Herein, a number of the basic parameters involved with the binding reaction between WEE virus and WS *Cx. tarsalis* BBF havae been optimized. Binding was found to be optimal at a pH of 7.2, 20^{0}C. No divalent cations are required for binding and equilibrium is reached within 1 hr.

Two basic criteria are of ultimate importance in demonstrating the physicochemical nature of specific binding of receptor (BBF) and ligand (WEE virus): saturation and competition. One must be able to demonstrate that in the presence of excess ligand essentially all receptors are occupied. Saturation of our system was reflected by the sigmoidal saturation curve for increasing amounts of input WEE virus versus WEE bound to a constant amount of BBF protein (Fig. 4). Similarly, where the Scatchard analysis of binding data intersects the x-axis (Fig. 5a), one finds saturation. For our system, saturation occurs at 5.9 μg WEE virus per μg BBF. Competitive displacement of radiolabeled virus was accomplished by increasing amounts of unlabelled virus (Fig. 6). Thus, the requirements of saturation and competitive displacement were satisfied and the specificity of interaction between WEE virus and BBF from susceptible *Cx. tarsalis* mosquitoes was established.

The binding of 5.9 μg WEE virus per μg BBF appears to be excessive. However as calculated above, this yields a rough estimate of 1.7-3.4 x 10^6 receptor sites/mesenteronal epithelial cell. This is very close to the number estimated for Sindbis virus in permissive cells (1.5 x 10^6; Smith and Tignor, 1980) and for 29S complexes of SF virus (1.3 x 10^6; Fries and Helenius, 1979). Similarly, the K_a for binding of WEE virus to BBF is only slightly higher (2.2 x 10^{11} M^{-1}) than that reported for SF virus binding to HeLa cells (3 x 10^{10} M^{-1}) or Eb cells (5 x 10^{10} M^{-1}) (Fries and Helenius, 1979).

2. *Monoclonal antibodies to brush border fragment proteins*

In the Annual Progress Report (1986), the production of a library of 95 monoclonal antibodies to Triton X-100® solubilized BBF from the WS and WR strains of *Cx. tarsalis* was reported. In the interim, the screening of these monoclonals has proceeded with two primary objectives. First, it was anticipated that a monoclonal antibody(ies) specific for the WEE viral receptor of WS *Cx. tarsalis* would be found. Second, the antigenic relatedness of the major proteins in the BBF from the WS and WR strains of *Cx. tarsalis* , was to be investigated. We have not begun to approach the former problem because of a lack of availability of highly purified and specifically monoclonal antibodies. We have attempted to approach the second problem by using all monoclonal antibodies that were highly specific for either WS or WR antigen in our initial screens (Table 6).

TABLE 6. Subcloning and ascites fluid production from hybridomas that revealed a high degree of specificity toward antigens from either WS or WR *Culex tarsalis* .

Antibody designation[1]	Ratio (WR:WS) of EIA rates as a measure of specificity[2]		Antibody subtype[3]	Subcloned	Ascites fluid
	Pre-freeze	Post-thaw			
1D4	∞	7:1	IgG_{2b}	**	
3D2	33:1	4:1	*		
12C4	1:99	2:1	*		
12D4	1:99	1:0.75	IgM	**	
13A5	1:76	1:0.08	IgG_{2b}	+	+
13B1	6:1	1:1	IgM + IgG_{2b}	**	
13C1	5:1	1:1.1	IgM	+	+
22A1	1:99	1:0.5	IgM + IgG_{2b}	**	
22A4	1:99	1:1.25	IgM + IgG_{2b}	**	
22A6	1:99	1:1.25	IgM + IgG_{2b}	**	

[1] The first number represents the growth plate which contained the hybridoma. The letter followed by a number is the cross-reference for the growth plate well that contained the hybridoma.

[2] Each hybridoma was screened against both WS and WR antigens and the ratio of EIA rates determined.

[3] The antibodies produced by the hybridomas were screened with a goat anti-mouse panel against IgG_1, IgG_{2a}, IgG_{2b}, and IgM.

* These hybridomas did not produce any detectable antibody beyond initial expansion into 24-well plates.

**These hybridomas did not survive beyond the 24-well plate stage.

Ten monoclonal antibodies were chosen to be thawed from storage in liquid nitrogen based on their initial EIA screenings that indicated they were highly specific for either WS or WR antigens, but not both. These hybridomas were grown to confluence in 24-well culture dishes and the supernatants tested for production of antibody specific for WS and/or WR BBF antigen with *Cx. pipiens* BBF proteins as a screen for nonspecific binding. The resultant EIA rates were extremely low. An antibody capture EIA assay indicated that all of the thawed antibodies were initially producing antibody (Fig. 7). In fact, the measured EIA rates were so low that an antibody capture assay was devised in order to verify that the hybridomas were producing antibodies (Fig. 7). The problem was partially resolved when Triton X-100® was removed from all wash solutions (See discussion below). A further complication arose when 2 hybridomas, 3D2 and 12C4, did not survive beyond the initial 24-well plate expansion (Table 6).

Antibody isotype determinations were completed on the supernatants from the initial 24-well plates (Table 6; Fig. 8). IgG_{2b} and IgM were the predominant isotypes within this

initial group of hybridomas. Some of the hybridomas were apparently producing some quantities of both IgG_{2b} and IgM (Table 6; Figs. 8F,H,I,J). This is not uncommon but it is a further complication if one wants to purify an antibody in order to prepare an immunoabsorbant column for the purification of specific mosquito antigens. Many of these hybridomas were apparently losing their ability to produce antibody; their EIA rates were extremely low (Fig. 8), compared to their rates upon initial isolation. The problem of how to deal with the dual antibody isotype producing hybridomas was resolved when we atempted to subclone the 8 remaining hybridomas. None hybridomas that were dual antibody producing continued to produce antibodies following subcloning. The 13A5 and 13C1 hybridomas were the only 2 of the original 10 that survived subcloning (Table 6).

The subcloning of 13A5 and 13C1 was by established methods. The hybridomas were grown to confluency in T-25 flasks, the cells were removed from the substrate, dispersed homogeneously in Iscove's MEM and a cell count determined. The cells were then distributed into a 96-well plates at the rate of 1 cell/ well. The cells were allowed to grow for 7-10 days at which time the yellowing cell culture fluid from those wells with growing colonies of cells were assayed for antibody production. The colony that yielded the highest EIA rate was expanded to a T-25 flask and the subcloning procedure repeated. At this point, a group of 13 subclones for 13A5 and 11 subclones of 13C1 were frozen in duplicate in liquid nitrogen.

A comparison was made between the ability of each of the subclones to detect antigen from either WS or WR mesenterons (Fig. 9). In virtually all cases, the WR antigen yielded the highest EIA rates (Fig. 9). This should be expected for the 13C1 monoclonal antibody (Fig. 9a), since it was derived from a mouse receiving only WR antigen. For 13A5, it could be surmised that the prevalence of the particular antigen detected appeared to be higher in WR mesenterons (Fig. 9B).

The freeze-thaw cycle caused some problems in continued production for some subclones. While all of the 13C1 subclones appeared to survive, only 7 of the original 15 subclones of 13A5 were still producing detectable antibody, after 2 or 3 passages in T-25 flasks, and 3 of these (13A5.4, 13A5.12 and 13A5.13) appeared to be diminishing in their capacity to produce antibody (Fig. 9).

An interesting complication arose as a consequence of trying to eliminate EIA background reaction rates through the inclusion of 0.05% Triton X-100® in the various wash solutions. The rates for the 13C1 hybridomas (Fig. 10A) were much lower that those for the

13A5 group (Fig. 10B). We then compared the EIA rates both with and without the addition of Triton X-100® (Fig. 10). The results conclusively demonstrated that the antigen detected by 13C1 was either eluted from the surface of the microtiter plate or blocked by the presence of Triton X-100® in the wash solution. There was no aapparent reduction in the EIA rates for 13A5 hybridomas.

An interesting adjunct to the observation with regard to the effects of Triton X-100® on the binding of mesenteronal antigens was previously reported (Final Report, 1987). In that study, the detection of antigen bound to nitrocellulose paper was examined following native blotting (Reinhart and Malamud, 1982). The most significant observation was that the mesenteronal antigens were primarily bound to the back side of the nitrocellulose paper. This suggested that some antigens had a higher affinity for the mobile phase from the isoelectric focused (IEF) acrylamide gel, which contained 0.5% Triton X-100®, than for the nitrocellulose paper. This might well be extended to include a higher affinity of some BBF antigens for Triton X-100® containing wash solutions than for the surface of the EIA plates.

Two subclones, one each from 13A5(.8) and 13C1(.6), were chosen to produce immune mouse ascitic fluid. We obtained about 50-60 ml of ascites fluid from those mice inoculated with 13C1.6 cells. Because this antibody is an IgM, no further purification of the antibody was attempted. From 13A5.8 ascitic fluid, we obtained approximately 35-40 mg of total antibody protein following ammonium sulfate fractionation and protein A column chromatographic purification. This is such a small quantity of antibody that we chose not to attempt to make a mini-affinity column to attempt to isolate specific mosquito antigens. Instead, we opted to wait for larger quantities of this particular monoclonal antibody to be made available from a bioreactor being tested by the campus Hybridoma Laboratory. The 13A5.8 subclone was chosen by the facility because it had been twice subcloned, it is quite stable and it has been well characterized. It will be used for the purpose of educating the laboratory's personnel in the set-up and maintenance of the system. In return, we are to be given the antibody produced in the bioreactor.

Three other hybridomas (19D1, 20C5 and 21B4), with no apparent differential affinities for either WS or WR antigens as determined by comparative EIA rates, were chosen for subcloning based on their high EIA rates in initial screenings. Again, survival of antibody production was a problem following thawing and expansion into 24-well plates. The 20C5 hybridoma was the only one of the three that survived. This particular hybridoma is an IgG_{2b} subtype and immune mouse ascitic fluid (200 ml) has been produced.

In collaboration with Dr. Alex Karu of the Hybridoma Laboratory, we agreed to an uncontrolled test of the effects of hydrocortisone treatment on ascites fluid production. Hydrocortisone treatment further immunosuppresses the irradiated mice and has been suggested to increase ascites fluid production. Six mice, out of a total of 24, were treated with hydrocortisone. One of the hydrocortisone treated mice yielded approximately 50 ml of ascites fluid over a 3 week period of collection; 1/4 of the total collected. The reported effects of hydrocortisone are enhanced production of ascites fluid over a longer period of collection. The results suggested that Dr. Karu should attempt to optimize the concentration and/or time of administration of hydrocortisone, under more carefully controlled conditions, in order verify the apparent stimulation of the volume of ascites fluid produced by the one mouse in our study.

3. *New monoclonal antibodies*

One of the major problems associated with the initial library of hybridomas is that, thus far, the antibodies screened have not been able to recognize denatured epitopes, separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and subsequently blotted onto nitrocellulose paper (Towbin et al., 1979). This makes differentiation of the major protein antigens associated with WS and WR BBF an impossibility because of the similarity of their pI's and molecular sizes. As a solution to this problem. we have begun the immunization of mice with individual proteins from WR and WS BBF, separated by two dimensional electrophoresis. The protein spots were sliced from the acrylamide gel, homogenized by passage through a series of needles of diminishing gauge in the presence of Freund's incomplete adjuvant, and inoculated in duplicate into two different strains of mice (Swiss-Webster and B10.Q). Initial tail bleed screenings indicate that the B10.Q strain has responded with more uniformly high rates that the Swiss-Webster strain (Data not shown), with a single exception. The production of hybridomas for the production of monoclonal antibodies is scheduled for the early part of October.

4. *Biochemical studies of brush border fragment proteins*

For a number of years, the significance of the major proteins associated with BBF from WS and WR *Cx. tarsalis* mesenterons in the binding of WEE virions has been a matter of speculation. It has been suggested that a high molecular weight protein found in WR BBF is posttranslationally modified in WS BBF, perhaps by proteolytic cleavage, into a lower molecular size protein (Ann. Prog. Rpt., 1985). It has been further suggested that this latter form of the protein might be involved in the binding of WEE virions to microvillar membranes.

Cx. pipiens is the refractory species in our interspecific model for the MIB to WEE virus. However, the 2D electrophoretic pattern of BBF from *Cx. pipiens* had not been analyzed. A pattern similar to that of WR *Cx. tarsalis* might further support the contention of involvement of this protein in binding. The BBF protein pattern for *Cx. pipiens* revealed two major proteins with very similar isoelectric points (pI's) but differing in their molecular weights (Fig. 11). This general pattern is exactly the same as that previously described for WR *Cx. tarsalis* (Ann. Prog. Rpt., 1985; 1986).

We continued to examine the distribution of the more hydrophobic proteins associated with BBF through high performance liquid chromatography (HPLC; Final Rpt., 1987). The major problem found in our previous studies was the extremely high ultraviolet absorption associated with Triton X-100®. Triton X-100® yields a number of spurious peaks in the HPLC separation of BBF proteins by reversed phase methods (Final Rpt., 1987). In addition, the magnitude of the peaks was such that protein peaks might easily be obscured. To attempt to circumvent this problem, the HPLC separation of BBF proteins was studied by solubilizing the BBF in two alternative nonionic detergents, N-octyl-glucopyranoside(OG), N-octyl-thioglucopyranoside(OTG).

The BBF proteins solubilized in OG and OTG, when separated by HPLC, revealed very minimal background (Figs. 12). In most cases, it was not apparent whether the minor peaks that appeared throughout the chromatogram were spurious or perhaps specific proteins associated with the BBF of that particular mosquito species/strain. Since their occurrence was not consistent either between mosquito strains/species or between detergents.

These same detergents (OG, OTG) were examined in native blots (Reinhart and Malamud, 1973) of horizontal IEF separations of BBF proteins. The proteins were found to bind primarily to the contact side of the support matrix (e.g., Immobilon® or nitrocellulose), as opposed to the noncontact side when Triton X-100® was used for solubilization (Data not shown). In the presence of Triton X-100®, BBF proteins appeared to have a greater affinity for the detergent phase than for the support matrix in the native blots.

It would appear that OG and OTG are superior detergents for the solubilization and subsequent analysis of BBF proteins, when compared to Triton X-100®. This appears to be especially true in analyses that are based on spectrophotometric analyses or blotting of proteins onto matrices that are based on hydrophobic interactions (e.g., Immobilon® and/or nitrocellulose).

B. Mesenteronal dissemination barrier-Selection of congenic strains for the modulation of WEE virus following parenteral infection

The congenic selection of an HVP producing strain of *Cx. tarsalis* from LVP parents has progressed very well. The selection process is designed to produce two groups of mosquitoes that are genetically identical except for a single locus (Groschel and Koprowski, 1965). In this case, the single gene is that for modulation of alphavirus titers. The results of the genetic selection of LVP *Cx. tarsalis* is summarized in Table 7. After 13 generations of backcrossing, the expected percent LVP in the population was in close agreement with the observed percent LVP ($\chi^2 = 2.99$, $p = 0.084$). The trend toward the production of a homozygous HVP *Cx. tarsalis* strain from our parental LVP line was very encouraging (Table 8). The results of 4 different brother-sister matings of progeny derived after 13 generations of selection demonstrate that the production of the homozygous HVP line has been achieved. Only one female, out of a total of 111 mosquitoes tested in group 4, revealed a titer of WEE virus in less than 4.0 $\log_{10}$ PFU/mosquito.

TABLE 7. Inoculation of western equine encephalomyelitis virus into parents from successive intercross - backcross matings of HVP and LVP *Culex tarsalis*.

Generation	Mating design	Genotype of mating	Viral replication: LVP/Total	Viral replication: Percent LVP[1] Observed	Viral replication: Percent LVP[1] Expected
	LVP Parent	pp	18/21	87	100
	$L.H_T$[2]	PP	0/31	0	0
G_0	Initial Cross	pp X PP	0/57	0	0
G_1	$Intercross_1$ [3]	Pp X Pp	17/82	26	25
G_2	$Backcross_2$ [4]	Pp X pp	33/86	39	33
G_3	$Intercross_2$	Pp X Pp	12/59	20[5]	25

1 If viral replication following inoculation controlled by a single dominant gene.
2 Designation of the presumed congenic, homozygous genotype for high virus production.
3 Mating inter se of HVP male and female offspring of previous cross.
4 Mating of LVP female to HVP male offspring from previous cross.
5 $\chi^2 = 2.99$, $p = 0.084$.

TABLE 8. Assessment of the progress toward selection of a homozygous line of HVP *Culex tarsalis* through successive brother-sister matings.

Brother/Sister	Males				Females			
Mating	Dose[1]	No.[2]	Mean[3]	LVP:HVP (%LVP)	Dose	No.	Mean	LVP:HVP (%LVP)
1	1.3	57	4.3	28:29 (49)	1.6	63	5.3	19:44 (29)
2	1.7	48	6.4	4:44 (8)	2.2	74	6.0	20:54 (27)
3	2.1	65	5.9	3:62 (5)	1.4	78	5.7	9:69 (12)
4	2.4	61	6.1	0:61 (0)	1.6	51	6.3	1:50 (2)

[1] Log_{10} PFU of WEE virus (BFS 1703, SMp2) inoculated intrathoracically per mosquito.
[2] Number of mosquitoes assayed.
[3] Mean log_{10} PFU WEE virus/mosquito.

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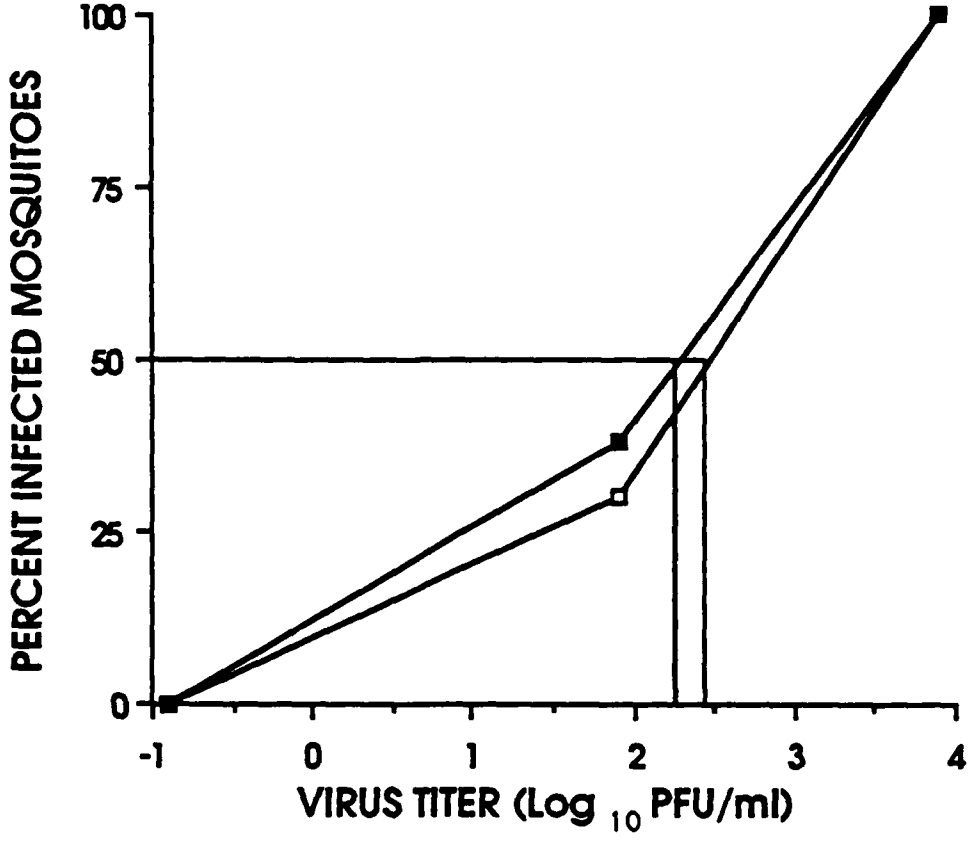

Figure 1. Graphic determination of the apparent ID_{50} following parenteral infection of the Rockefeller strain of *Aedes aegypti*. 7 days (□) and 10 days (■) incubation at 32°C following intrathoracic inoculation.

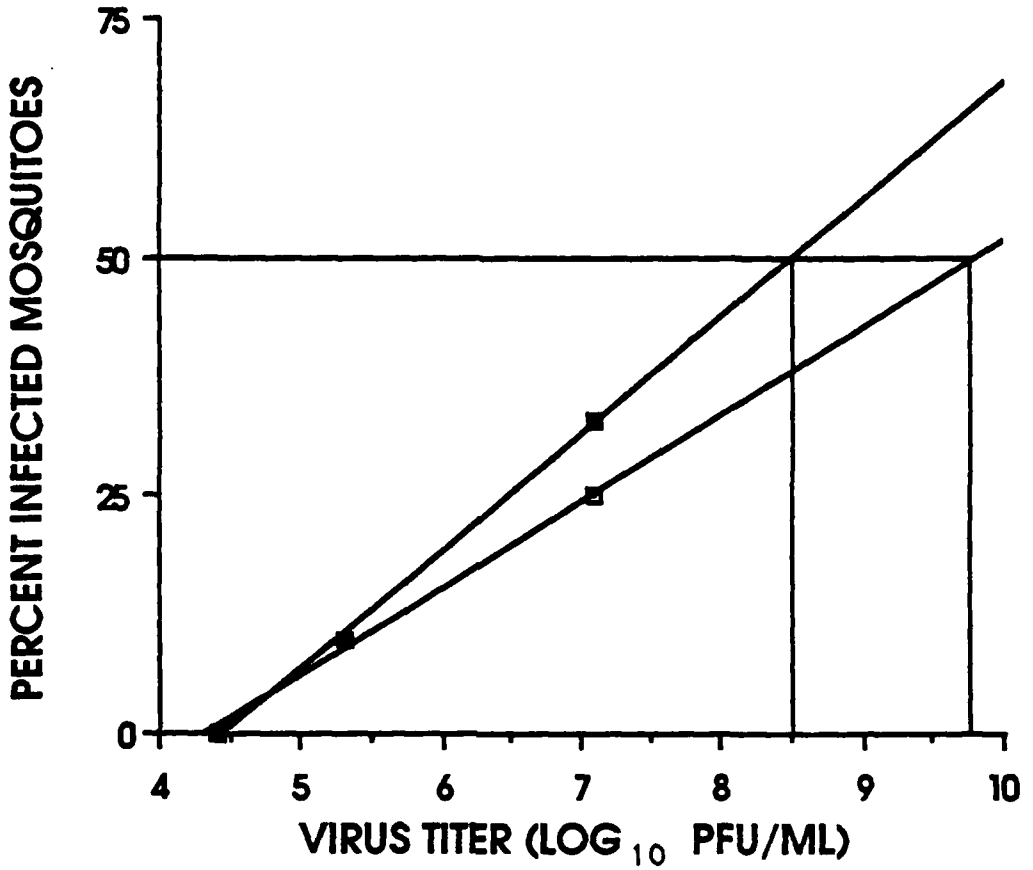

Figure 2. Graphic determination of the apparent ID_{50} following peroral infection of the Rockefeller strain of *Aedes aegypti*. 11 days (▭) and 14 days (■) incubation at 32°C following peroral infection.

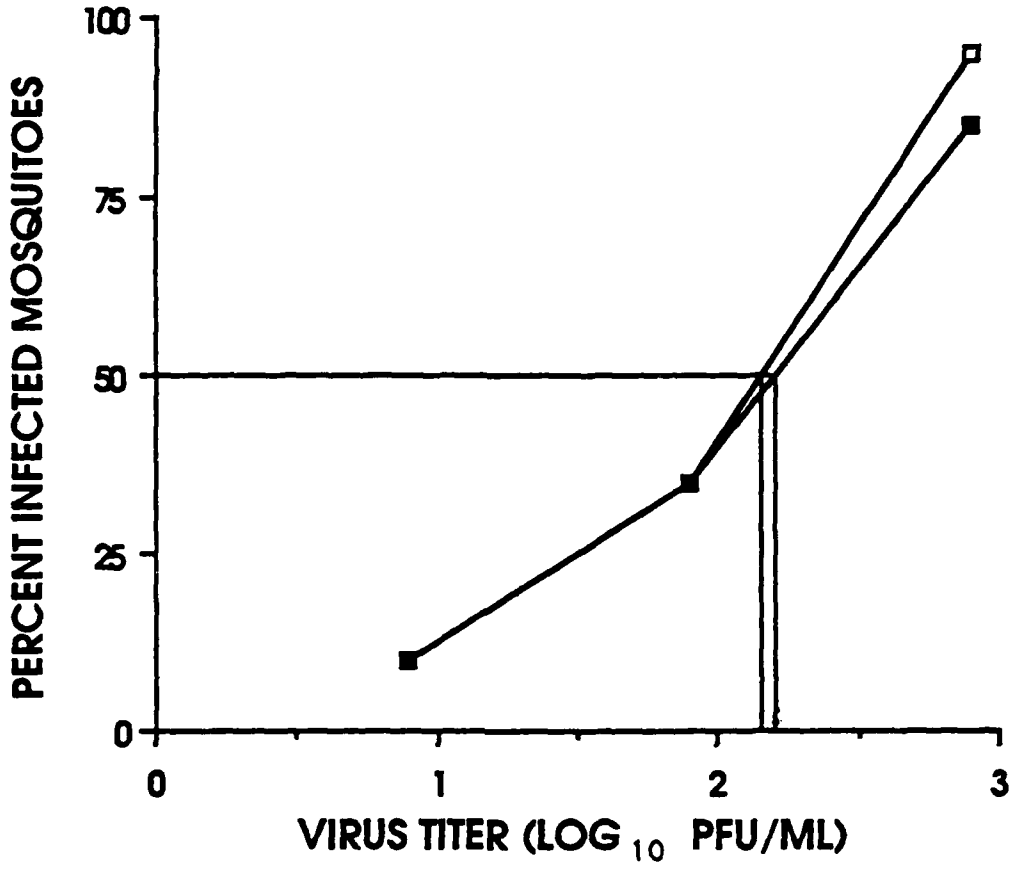

Figure 3. Graphic determination of the apparent ID_{50} following parenteral infection of the Jupp strain of *Aedes aegypti*. 7 days (□) and 10 days (■) incubation at 32°C following intrathoracic inoculation.

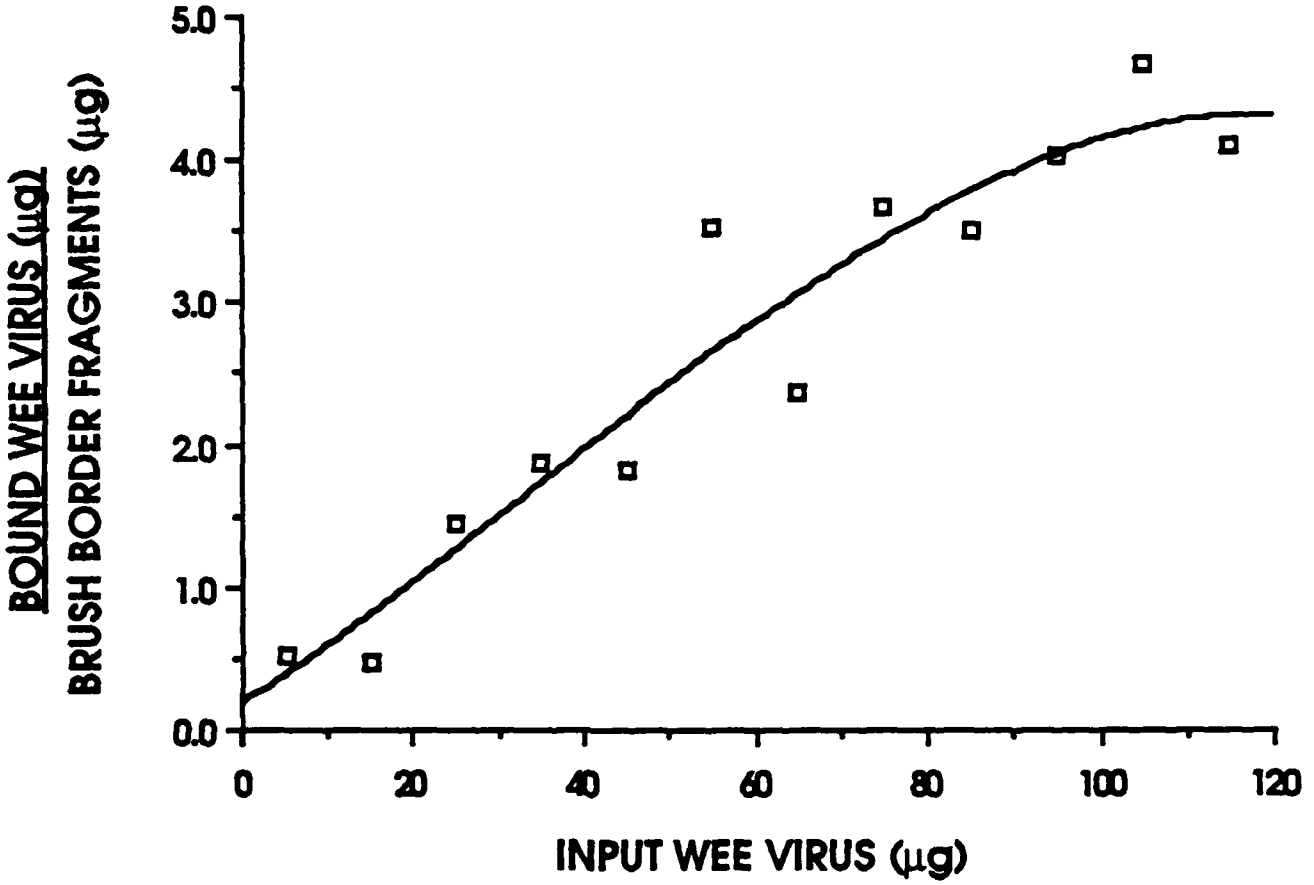

Figure 4. Saturation of WEE viral receptor sites on the BBF isolated from the mesenteronal epithelial cells of the WS strain of *Culex tarsalis* . The data were combined from 5 separate experiments (r=0.96).

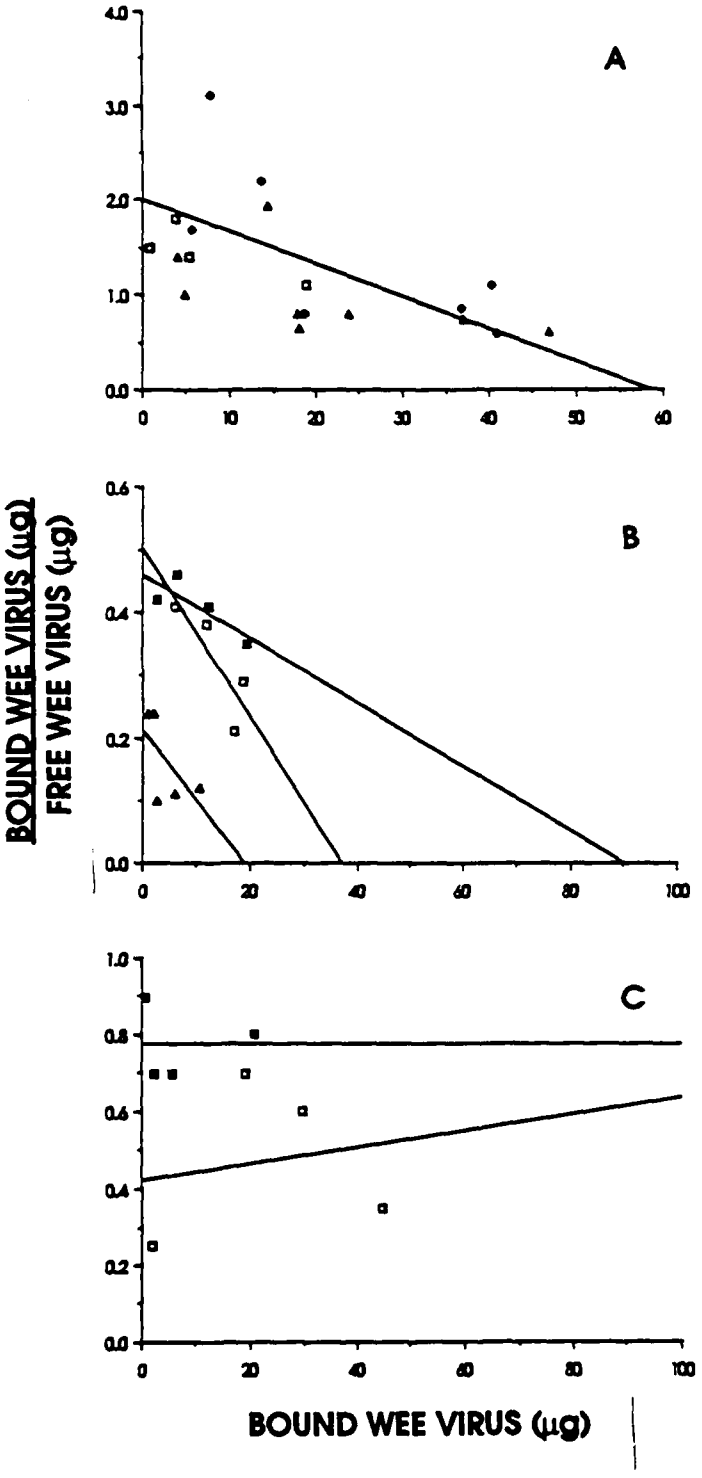

Figure 5. Scatchard analyses of the binding data for (A) WS *Culex tarsalis* , (B) *Culex pipiens* , and (C) WR *Cx. tarsalis* . The line of best fit for the WS *Cx. tarsalis* ($r=0.76$; $t_{0.01}=2.9<<4.75$) yields complete saturation of binding sites at 5.9 µg WEE virus/µg BBF, 5.5×10^{10} binding sites/µg BBF and an affinity constant of 2.3×10^{11} M^{-1}.

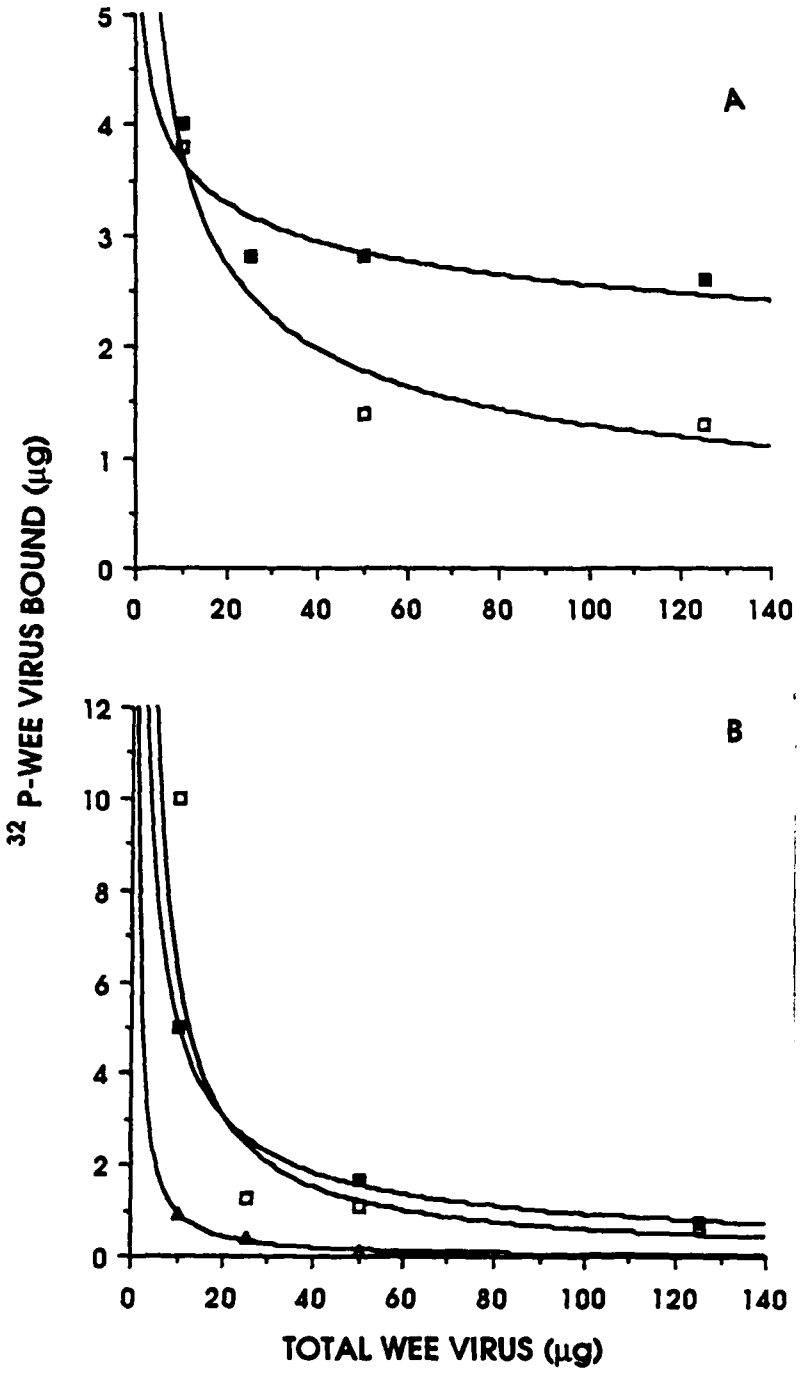

Figure 6. Competitive binding between ^{32}P-WEE and unlabelled WEE virus for receptor sites on BBF. (A) Comparison between *Culex pipiens* (■) and WS *Culex tarsalis* (□). (B) Results of three competitive binding experiments where the specific binding to WS *Cx. tarsalis* BBF is corrected for the background binding of *Cx. pipiens* . (□, r=0.90; △, r=0.99; ■, r=1.00).

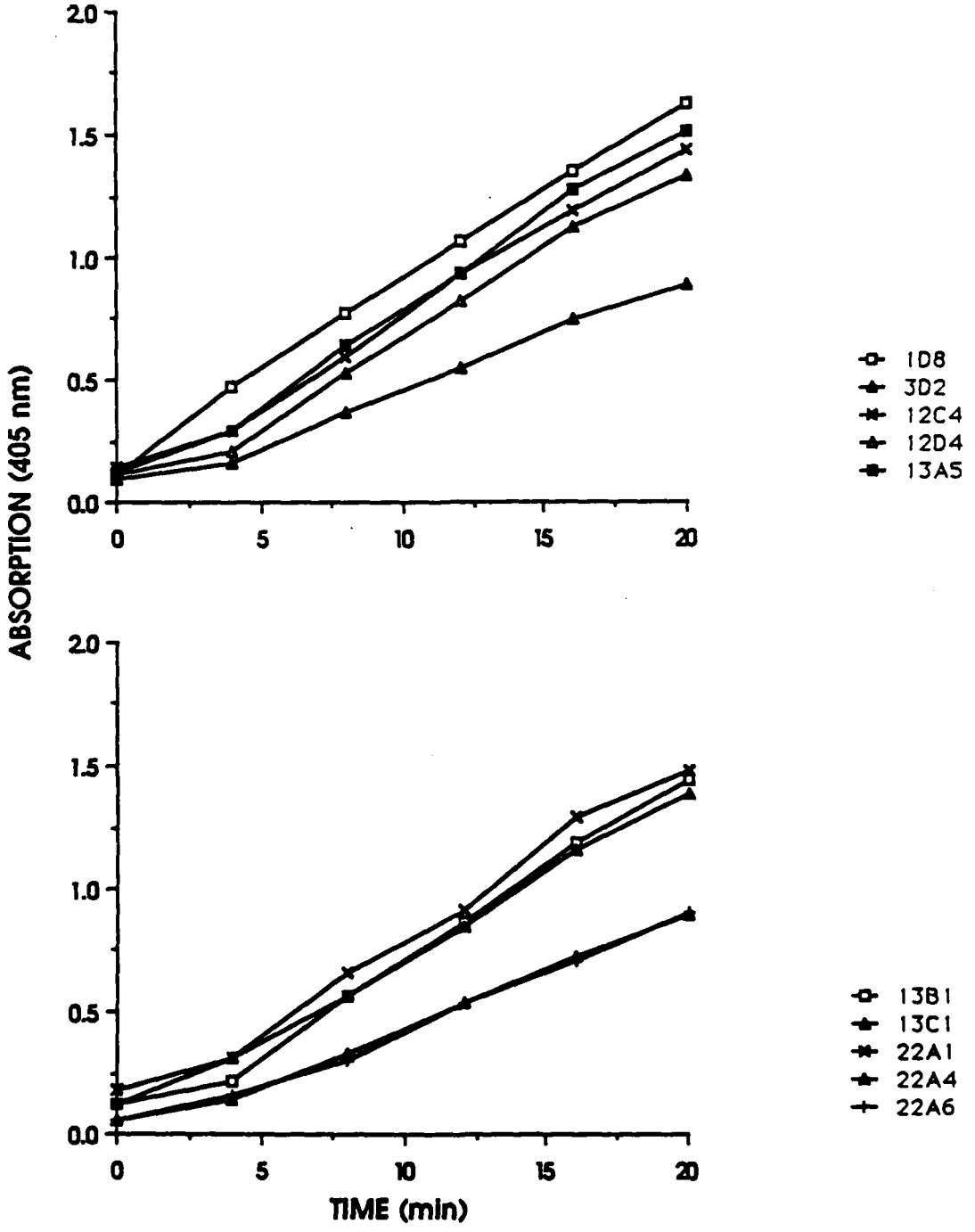

Figure 7. Antibody capture EIA assay to determine if thawed hybridomas were producing antibody to BBF from the mesenterons of *Cx. tarsalis* .

Figure 8. Antibody isotype determination for 10 hybridomas screened for potential subcloning. (A) 1D8, (B) 3D2, (C) 12C4, (D) 12D4, (E) 13A5, (F) 13B1, (G) 13C1, (H) 22A1, (I) 22A4 and (J) 22A6.

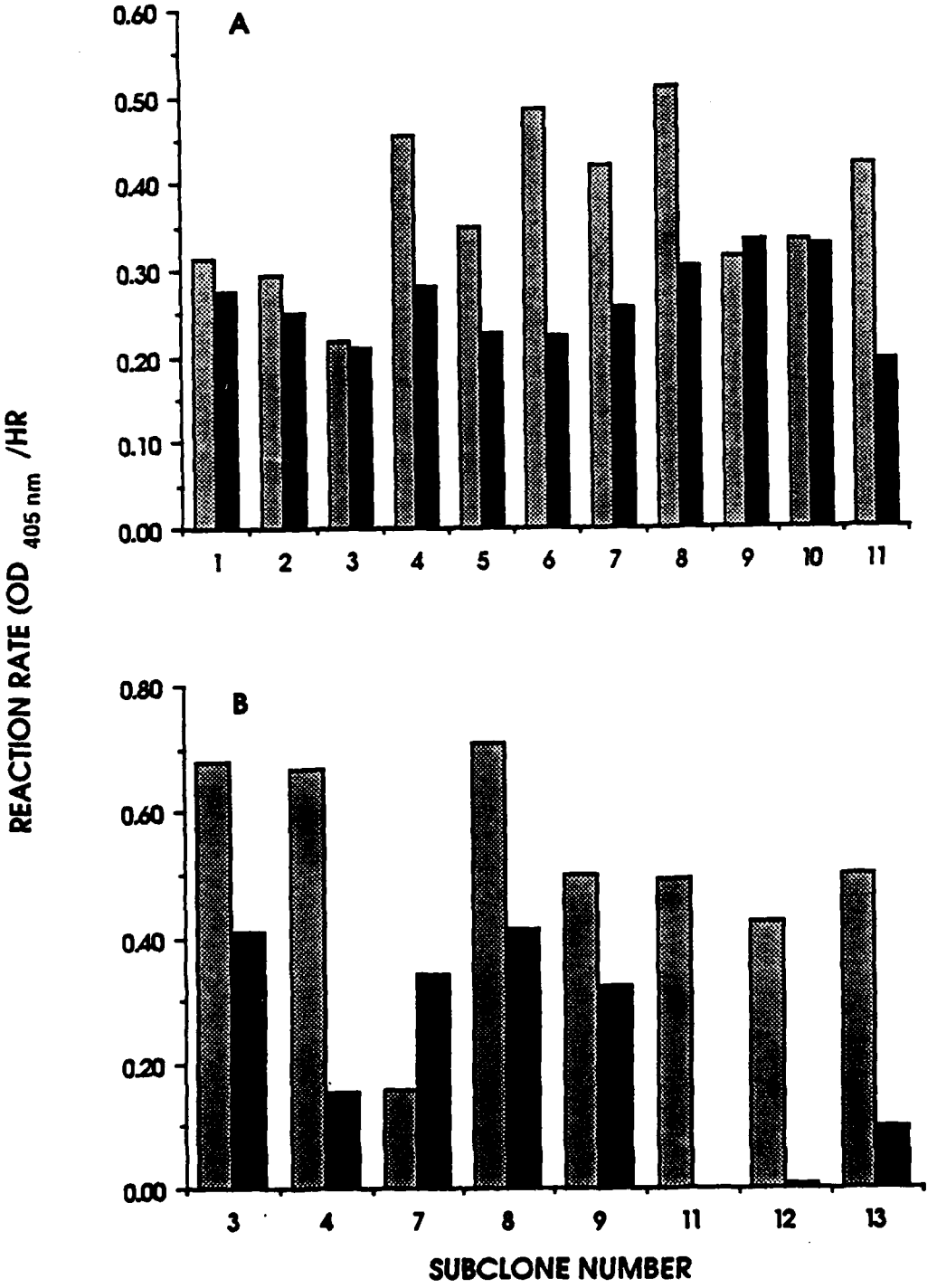

Figure 9. Comparison of the EIA rates of 13C1 (A) and 13A5 (B) subclones for antigen derived from either WR (low density bars) or WS (high density bars) *Culex tarsalis* mesenterons.

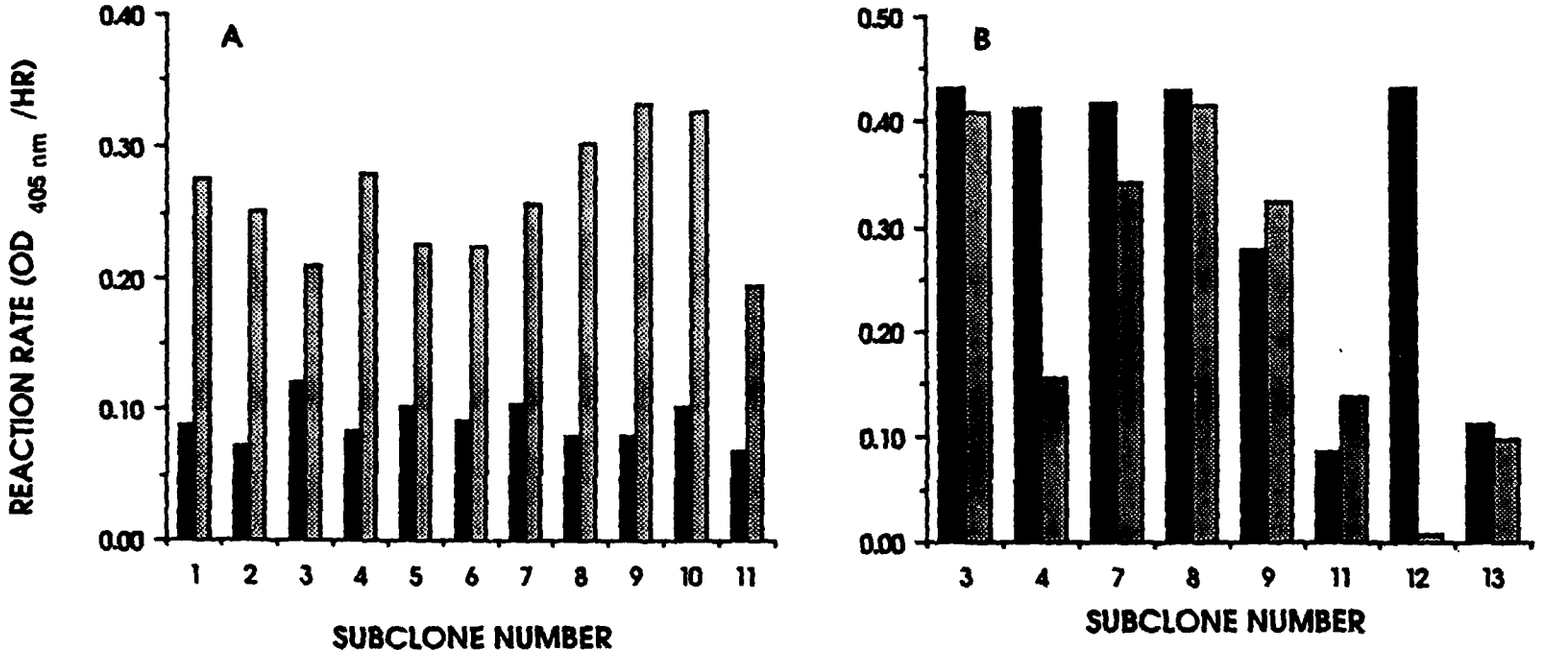

Figure 10. The effects of Triton X-100® on the EIA rates for various subclones of the 13C1 and 13A5 hybridomas. Low density bars are the rates without Triton X-100® and the high density bars are the rates with Triton X-100® in the wash solutions.

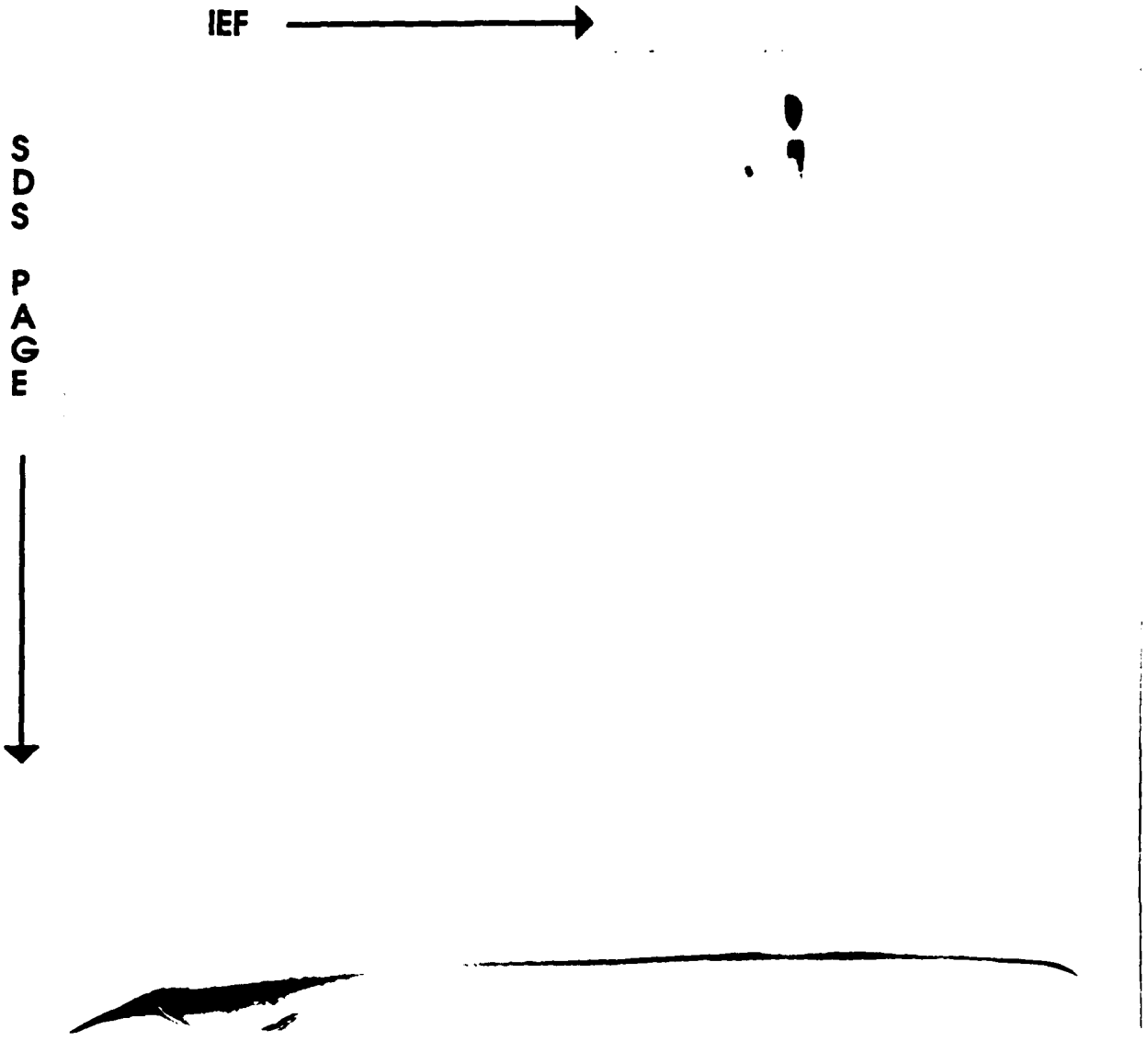

Figure 11. Two dimensional separation of mesenteronal epithelial cell BBF proteins from *Culex pipiens* .

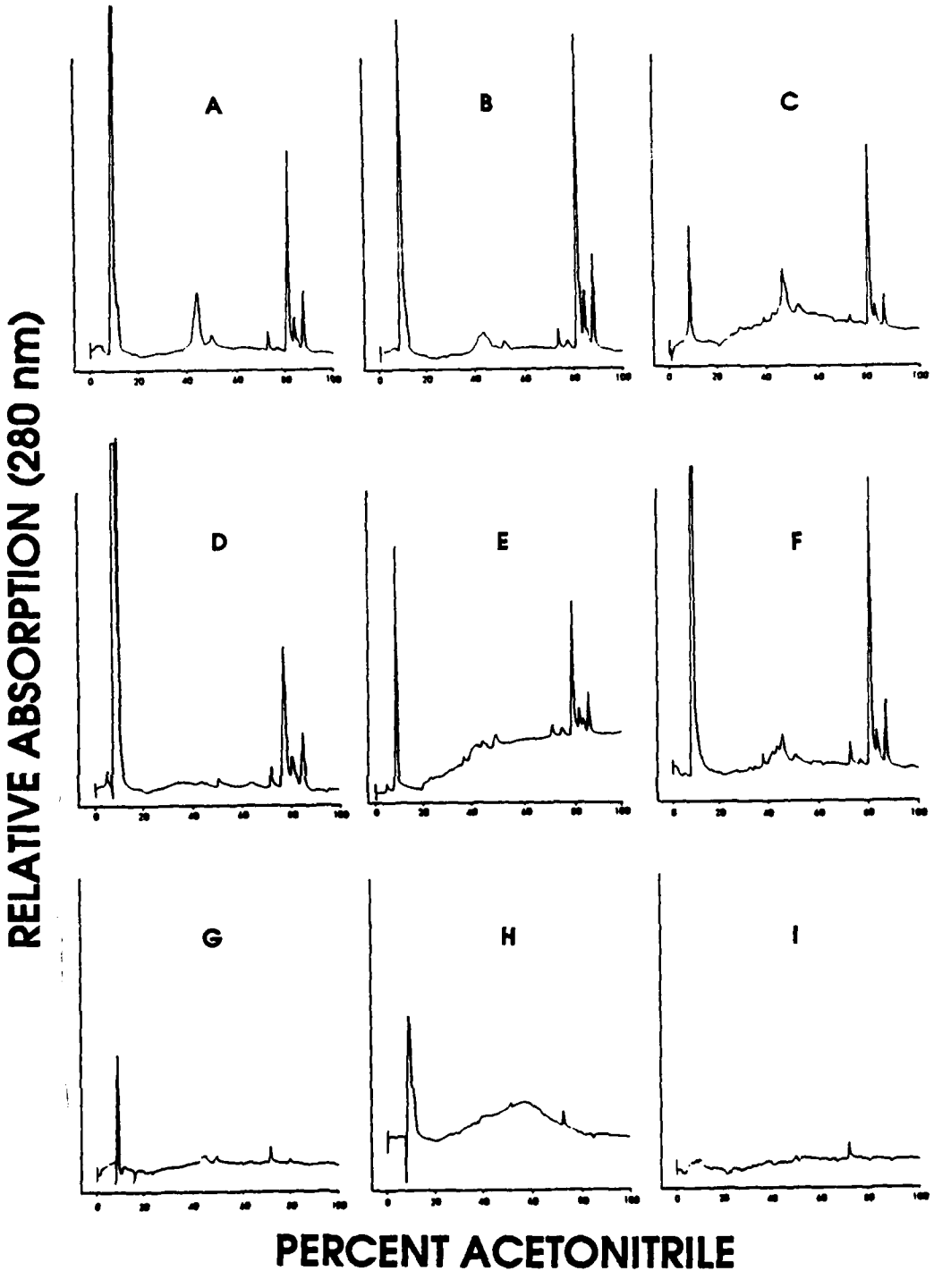

Figure 12. Reversed phase high performance liquid chromatography of BBF proteins from the mesenteronal epithelial cells of WS (A, D) and WR (B, E) *Culex tarsalis* and *Culex pipiens* (C, F). The BBF proteins were solubilized with either n-octyl glucoside (A, B, C) or n-octyl thioglucoside (D, E, F) and separated by gradient elution in acetonitrile (0-100% v/v) with 0.1% trifluoroacetic acid. Detergent blanks for n-octyl glucoside (G) and n-octyl thioglucoside (H); solvent blank (I).

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